# Role of Hippo and Ecdysone Receptor Signaling in regulation of *dronc*

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## Abstract

The Hippo pathway is an evolutionarily conserved pathway that regulates organ size and tissue homeostasis in *Drosophila* and mammals. The pathway functions by regulating the nuclear availability of transcriptional cofactor Yorkie (Yki), mammalian YAP, which is regulated by the activity of a core kinase cascade comprising the serine threonine kinases Hippo (Hpo) and Warts (Wts) and their accessory proteins. Yki binds with transcription factors like Scalloped (Sd) or Homothorax (Hth) to regulate target genes involved in cell proliferation and survival. Downregulation of the Hpo pathway causes increased cell proliferation and overgrowth, whereas hyperactivation of this pathway leads to cell death due to activation of caspases. Caspase proteins are cysteine aspartic proteases which play essential roles in cellular signaling and development via apoptosis. We showed that the initiator caspase *dronc* (mammalian Caspase 9) is a transcriptional target of Yki. We found that loss of Hippo signaling leads to downregulation of *dronc* expression, whereas downregulation of Sd resulted in upregulation of *dronc* expression. We also found that known binding partner of Sd like E2F1 is also involved in regulating *dronc* expression. Earlier studies have shown that dronc expression is regulated by the Ecdysone receptor (EcR) signaling pathway and mapped an EcR regulatory element on *dronc* promoter. We found that depletion of EcR or its corepressors like Smrter caused upregulation of *dronc* expression. Overexpression of Taiman (Tai) a binding partner of EcR and Yki also upregulated *dronc* expression. We also show that Tai-Yki interaction may not be required for *dronc* regulation. We hypothesize that *dronc* expression is regulated by the Hippo and EcR signaling pathways. Here, we present our work on the regulation of *dronc* by the Hippo and EcR signaling pathways, and its implications on development.

## Introduction

Current research is focused on how Hippo pathway regulates cell proliferation and cell death, and how interactions with other pathways modify the outputs of the Hippo pathway in normal cells and in cancer. The core components of the pathway include two serine-threonine kinases Hippo (Hpo) and its target Warts (Wts), and the transcriptional co-activator Yorkie (Yki). When the pathway kinases are active, Hpo along with Wts and cognate adaptor proteins Salvador (Sav) and Mob as Tumor Suppressor (Mats) bring about phosphorylation of Yki which leads to its cytoplasmic sequestration and cell death. Loss of function of the pathway promotes the formation pf an activator complex between the non-phosphorylated Yki and the transcription factor Scalloped (Sd) to regulate target gene expression of cell cycle and cell proliferation genes such as Cyclin E, A, B, D, and *drosophila inhibitor of apoptosis* (*diap1*); and downregulation of *dronc* (*Drosophila* Nedd-2 like caspase; an orthologue of human initiator caspase- Caspase-9), and effector Caspase-3 homologue, *drice* in *Drosophila* (Verghese et al 2012). Development of *Drosophila*, molting and metamorphosis is controlled by short pulses of elevated levels of the steroid hormone, ecdysone. High pulses of ecdysone expression are correlated with increased Dronc activation, which causes programmed cell death (PCD). **Ecdysone works through its heterodimeric receptor EcR-Usp** to regulate gene expression. EcR can bind to a binding element (EcRBE) of *dronc* promoter and based on the co-factor/protein bound to EcR, it can act as an activator or a repressor (Dorstyn et al 1999, Daish e al 2003, Cakouras et al 2004). **However, how EcR and Yki regulate *dronc* expression remains unclear.** Our aim is to find out the mechanism of this regulatory interaction.

**Figure 1: Schematic diagram of core kinase components of Hippo Pathway and *dronc-lacZ* constructs**

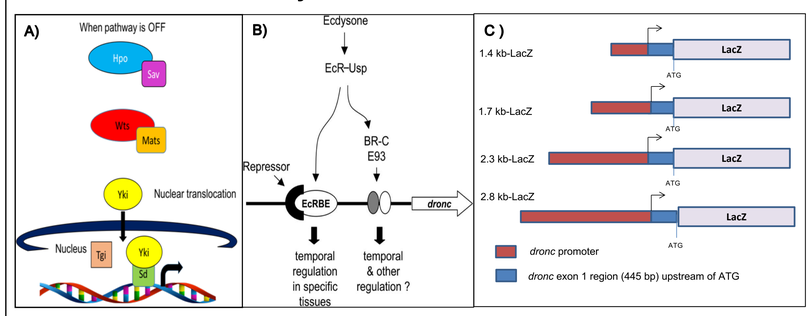


**Figure 1: A) Core kinase cascade in the Hippo pathway in *Drosophila.*** Cartoon shows interactions among pathway components when Hippo signaling is OFF (right). Downregulation of the Hippo pathway results in release of Wts mediated inhibition of Yki, which can bind with its cognate transcription factors, *e.g.*, Scalloped (Sd), and translocates to the nucleus to control expression of Hippo target genes. **B) Cartoon showing Ecdysone mediated *dronc* regulation** by its heterodimeric receptor EcR-USP and Ecdysone inducible genes BR-C/E93. Binding of a potential repressor can lead to spatio-temporal expression of *dronc* (Cakouras et. al., 2004) **C) *dronc* promoter constructs:** Cartoon depicts the different deletion constructs of the *dronc* promoter used in this study. Various lengths of the promoter upstream of the *dronc* transcription start site were cloned upstream of a LacZ reporter gene. (Flies made by Dr. S. Kumar)

**Figure 2: Effect of downregulation of *wts* on *dronc* promoters**

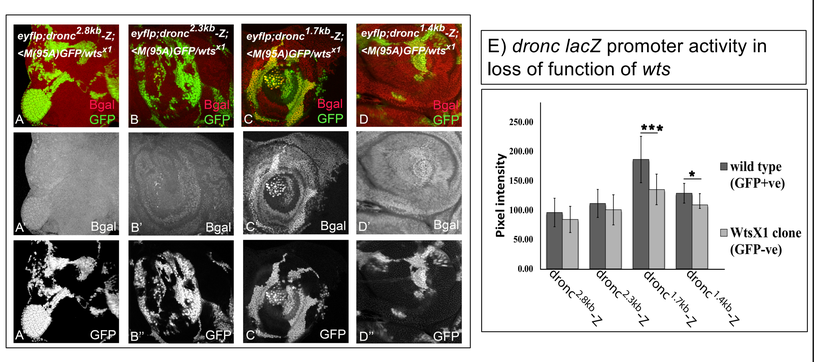


**Figure 2: Downregulation of *wts* causes reduction in *dronc* expression.** Panels show effects of loss of $wts^{X1}$ on the levels of (A-A'') full length $dronc^{2.8kb}$-*lacZ* promoter, or its deletion constructs: (B-B'') $dronc^{2.3kb}$-*lacZ*, (C-C'') $dronc^{1.7kb}$-*lacZ* and (D-D'') $dronc^{1.4kb}$-*lacZ*. E) Quantification of pixel intensity for Wild-Type (GFP positive, shown as dark grey bars) versus $wts^{X1}$ mutant clones (GFP negative, shown as light grey bars) is shown. Fold change is plotted as % ± SD . p-values: *p<0.05 and ***p< 0.001, n = 20.

**Figure 3: Effect of Yorkie on *dronc1.7* expression**

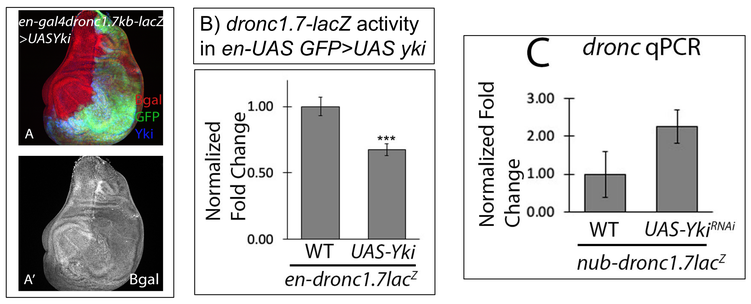


**Figure 3: Upregulation of *yki* causes repression of *dronc* expression and downregulation of Yki induces *dronc* transcription.** (A-A') Panels show wing imaginal discs from *engrailed gal4 UASGFP UASYki* larvae (posterior compartment, GFP+ve). Wild type is represented by anterior compartment. B) Effects of gain of Yki on the levels of $dronc^{1.7kb}$-*lacZ* are plotted as fold change% ± SD for Wild-Type versus *UAS yki*. p-values: ***p<0.001. n = 5 C) qPCR of *dronc* in loss of Yki. Normalized fold change expression are calculated using ddCt method. n = 5.

**Figure 4: Role of Yki/Sd in growth control and *dronc* expression**

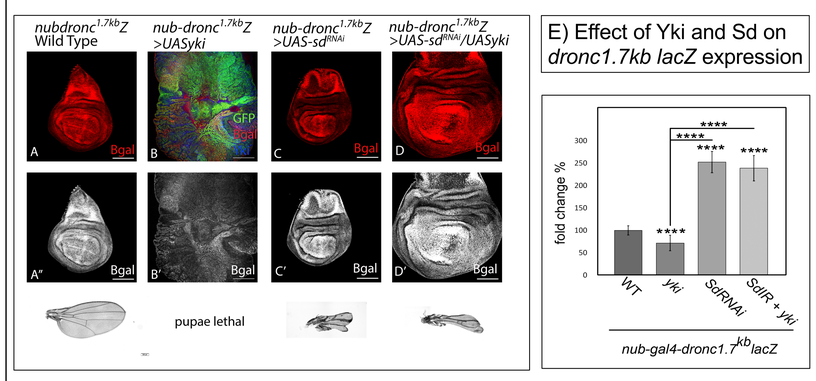


**Figure 4: Loss of Sd limits Yki induced overgrowth and causes *dronc* de-repression.** Panels show effects of loss of Sd on growth and *dronc* regulation by Yki in wing discs from (A-A') *nub-Gal4* that show Wild-Type levels of $dronc^{1.7kb}$-*lac-Z*, (B-B') *nubGAL4 UAS yki*, (C-C') *nubGAL4 UAS-*$sd^{RNAi}$, (D-D') *nubGAL4 UASyki UAS-*$sd^{RNAi}$. E) Plotted is fold change % ± SD. p-values: ****p<0.0001. n = 20

**Figure 5: Effect of loss of EcR and Sd on *dronc* expression**

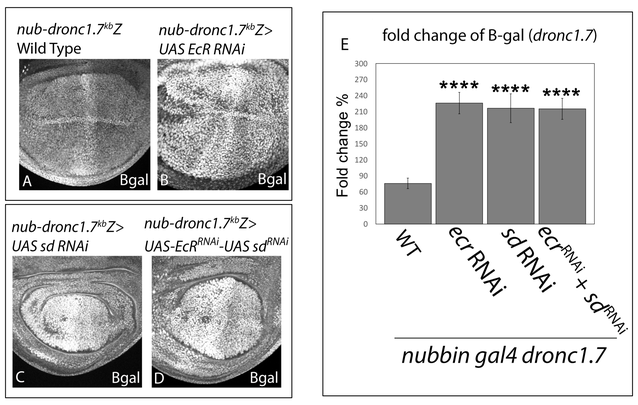


**Figure 5: EcR and Sd limit growth and keep *dronc* levels in check.** Panels show a comparison of $dronc^{1.7kb}$-*lac-Z* expression in wing discs from larvae of the following genotypes: (A) *nub-GAL4* $dronc^{1.7kb}$-*lacZ*, (B) *nub-GAL4* $dronc^{1.7kb}$-*lacZ UAS-*$ecr^{RNAi}$, (C) *nub-GAL4* $dronc^{1.7kb}$-*lacZ UAS-*$sd^{RNAi}$ and (D) *nub-GAL4* $dronc^{1.7kb}$-*lacZ UAS-*$ecr^{RNAi}$ *UAS-*$sd^{RNAi}$. E) Plotted is fold change % ± SD. p-values: ****p<0.0001. n = 20

**Figure 6: Effect of Yki and Ecr on $dronc^{1.7kb}$ promoter**

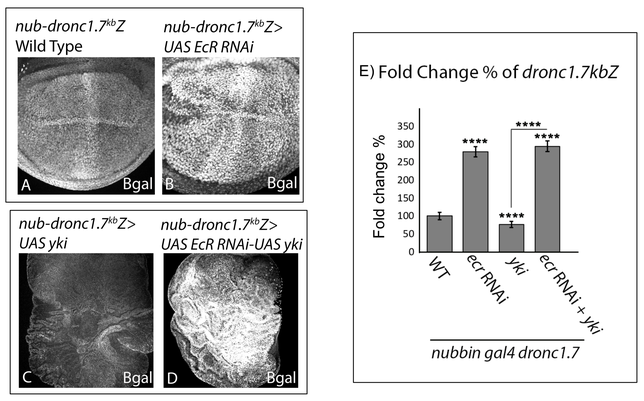


**Figure 6: Yki and EcR keep *dronc* levels in check.** Panels show a comparison of $dronc^{1.7kb}$-*lac-Z* expression in wing discs from larvae of the following genotypes: (A) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z*, (B) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$ecr^{RNAi}$, (C) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS yki* and (D) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$ecr^{RNAi}$ *UAS-yki*. E) Plotted is fold change % ± SD. p-values: ****p<0.0001. n = 20

**Figure 7: Effect of Taiman misexpression on $dronc^{1.7kb}$ reporter**

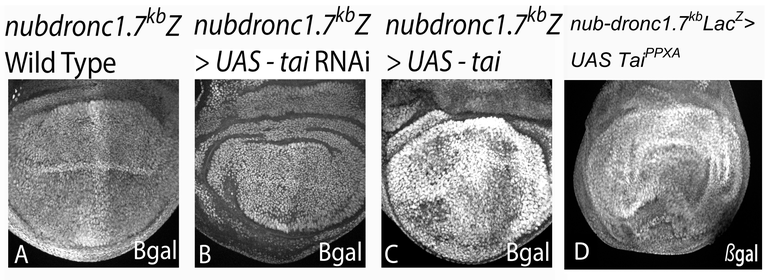


**Figure 7: Taiman regulates *dronc* expression.** Panels show a comparison of $dronc^{1.7kb}$-*lac-Z* expression in wing discs from larvae of the following genotypes: (A) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z*, (B) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$tai^{RNAi}$, (C) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS tai,* and D) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$tai^{PPXA}$. Down-regulation of *tai* leads to repression of *dronc* and upregulation of Taiman induces *dronc* expression. Taiman does not require Yki interaction to regulate *dronc.*

**Figure 8: Effect of Tai-Sd interaction on $dronc^{1.7kb}$ *lacZ***

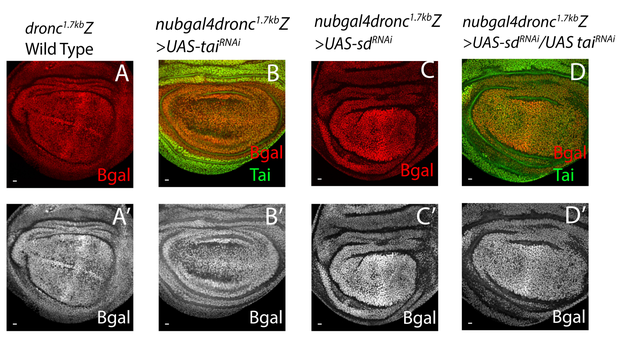


**Figure 8: Loss of Sd upregulates *dronc* and loss of Taiman downregulates *dronc*.** Panels show a comparison of $dronc^{1.7kb}$-*lac-Z* expression in wing discs from larvae of the following genotypes: (A) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z*, (B) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$Tai^{RNAi}$, (C) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$Sd^{RNAi}$ and (D) *nub-GAL4* $dronc^{1.7kb}$-*lac-Z UAS-*$sd^{RNAi}$/$Tai^{RNAi}$. Down-regulation of Sd and Tai together leads to induction of *dronc.*

## Methods:

- IHC was performed using antibodies against $\beta$ – galactosidase.
- The quantification was done using Photoshop CS6 and MS Excel (students T-test, unequal variances, $\alpha$ = 0.05)

## Conclusion:

- Loss of *wts* or over-expression of Yki cause a significant downregulation of the full-length 2.8kb-dronc promoter and its deletion constructs (2.3kb, 1.7kb and 1.4kb, fig 2, 3)
- Downregulation of *sd* showed a reduction in wing pouch size and strong *dronc* derepression (Figure 4) Yki induced overgrowth is suppressed by downregulation of Sd, and *dronc* expression is derepressed suggesting that Yki requires Sd to regulate growth and to limit the inappropriate expression of *dronc* (Fig. 4).
- Downregulation of EcR and Sd showed strong *dronc* derepression and a reduced wing phenotype suggesting that Sd may be epistatic or synergistic to EcR, however, how EcR and Sd regulate *dronc* expression is yet to be determined (Fig 5).
- We found that Yki fails to repress *dronc* in absence of EcR suggesting requirement of EcR in suppressing *dronc* expression (Fig. 6). Further, EcR is not required for Yki mediated growth regulation, suggesting that Yki regulates growth independently of dronc regulation.
- Loss of Taiman suppresses *dronc* expression whereas its upregulation induces *dronc* expression ( Fig. 7)
- Sd acts downstream of Tai in repressing *dronc* expression (Fig. 8)

## Future Directions:

We will perform genetic and biochemical analyses to:

- Test if Sd acts epistatic to EcR or synergistically in regulating *dronc* expression.
- Identify the relation between Yki and EcR in regulating *dronc* expression.
- Identify the mechanism by which Hippo pathway and Ecdysone pathway regulate *dronc*

## References:

•Verghese, S., S. Bedi, and M. Kango-Singh. 2012. "Hippo Signaling Controls Dronc Activity to Regulate Organ Size in Drosophila." *Cell Death and Differentiation* 19 (10): 1664–76. https://doi.org/10.1038/cdd.2012.48

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